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Detection of a human intracisternal retroviral particle associated with CD4+ T-cell deficiency

(*Pneumocystis carinii* pneumonia/human immunodeficiency virus-negative AIDS)

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ABSTRACT **A number of non-human-immunodeficiency-virus (HIV) type 1 disorders are associated with CD4+ T-cell deficiency and dysfunction. However, the etiopathogenesis of CD4+ T-cell immunodeficiency in these disease states remains unclear. Human intracisternal retroviral (HICRV) particles were detected in a lymphoblastoid cell line exposed to mononuclear cells from a patient with severe CD4+ T-cell deficiency without risk factors for HIV infection. Ultrastructurally, the HICRV is distinct from HIV-1, HIV-2, human T-lymphotropic virus (HTLV) type I, and HTLV-II. Supernatants of activated mononuclear cells showed significant reverse transcriptase activity that was predominantly Mn^{2+} dependent. The patient's mononuclear cells were negative for HIV-1, HIV-2, HTLV-I, and HTLV-II proviruses as demonstrated by the lack of amplification by PCR. Also, the patient's serum was negative for antibodies to HIV-1, HTLV-I, and HTLV-II and for HIV-1 p24 antigen; however, serum was positive for antibodies against the HICRV as demonstrated by Western blot. Similar HICRV particles were detected in a lymphoblastoid cell line exposed to mononuclear cells from the patient's daughter, who showed CD4+ T-cell dysfunction. The HICRV may be associated with CD4+ T-cell immunodeficiency and dysfunction in patients without risk for HIV-1, HIV-2, HTLV-I, and HTLV-II.**

Human retroviruses are associated with several human diseases that involve disturbances of the growth of CD4+ T lymphocytes (1). Human T-lymphotropic virus (HTLV) type I is associated with malignant expansion of CD4+ T cells (2), whereas human immunodeficiency virus (HIV) type 1 is associated with depletion of CD4+ T cells, resulting in AIDS (3–5). Recently, a human intracisternal A-type retroviral particle was isolated from lymphoblastoid cells exposed to homogenates of salivary gland from Sjögren syndrome (6). The latter virus was antigenically related to HIV-1 p24. No relationship to CD4+ T-cell deficiency was reported. A number of non-HIV-1 disease states are associated with CD4+ T-cell deficiency (7–17). Jacobs *et al.* (18) have described a cluster of HIV-1-negative patients with *Pneumocystis carinii* pneumonia without predisposing illness, some of whom had T-cell defects. More recently, Gautier *et al.* (19) reported three cases of *P. carinii* pneumonia associated with CD4+ T-cell deficiency. No HIV-1 antibody or HIV-1 antigen was found during 1 year of follow-up. However, the etiopathogenesis of immune deficiency in these subjects remains unclear. In the present report, we demonstrate the presence of a human intracisternal retroviral (HICRV) particle in a lymphoblastoid cell line (H9) and in peripheral blood mononuclear cells (MNC) from a healthy control that were exposed to MNC from a 66-year-old patient who had a severe CD4+ T-cell deficiency (Table 1) and *P. carinii* pneumonia but had no risk factors for HIV infection. Similar HICRV particles were demonstrated in cocultures of H9 cells and the MNC of this patient's asymptomatic daughter who displayed CD4+ T-cell dysfunction. Ultrastructurally, enzymatically, antigenically, and by PCR, HICRV is distinct from HIV-1, HIV-2, HTLV-I, HTLV-II, and from the human intracisternal A-type retroviral particle.

MATERIALS AND METHODS

Materials. Recombinant interleukin-2 (rIL-2) was purchased from Amgen. A thermal cycler, primers SK145, SK431, SK43, and SK44, and a GeneAmp PCR kit were purchased from Perkin–Elmer/Cetus. Synthetic templates, dT_{12-18}·poly(rA) and dG_{12-18}·poly(rC), for reverse transcriptase (RT) activity were purchased from Pharmacia. U1 cells, a subclone of U937 cells that were infected with HIV-1 (20), and MT-2 cells (21), a T-cell line infected with HTLV-I, were obtained from AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Monoclonal antibodies to HIV-1 p24 were purchased from Dako (Carpinteria, CA). Western blot detection kits were purchased from Amersham.

Coculture of Patient's MNC with H9 Cells and MNC from Controls. MNC (2×10^6 per ml) from the patient (no. 200) were suspended in RPMI 1640 supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum, hereafter termed culture medium, and cultured with phytohemagglutinin (PHA) P at 10 μg/ml for 72 hr at 37°C in a 5% CO_2/95% air atmosphere. The medium was then changed, and cells were resuspended in culture medium containing rIL-2 at 10 units/ml. At day 7, cells were cocultured with H9 cells or MNC from a healthy control that were preactivated with PHA for 3 days, at a ratio of 1:3 (patient/H9 MNC or patient/normal control MNC). The culture medium was changed every 3 days, and cells were examined for syncytia formation and cell viability. After 3 weeks of culture, cells were analyzed for virus particles by electron microscopy. For RT activity, supernatants were collected at various time intervals from PHA- plus rIL-2-activated MNC of the patient and cocultures of the patient's MNC and H9 cells (H9/200) and frozen at −70°C until analyzed.

Electron Microscopy. Cells were pelleted, fixed in cold 2.5% glutaraldehyde in 0.12 M phosphate buffer (pH 7.4) for 30 min, and rinsed in buffer prior to postfixation in 1% osmium tetroxide for 30 min. The pellets were embedded in 4% Bacto-agar solution to hold them together during processing. Blocks were made by using a razor blade and dehydrated with ethyl alcohol and propylene oxide before being embedded in Medcast. Ultrathin sections of these

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Abbreviations: HIV, human immunodeficiency virus; HTLV, human T-lymphotropic virus; PHA, phytohemagglutinin; rIL-2, recombinant interleukin 2; HICRV, human intracisternal retrovirus; MNC, peripheral blood mononuclear cells; RT, reverse transcriptase; SSV, simian sarcoma virus.

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Table 1. Immunological analysis of the patient (no. 200)

Tests	Value	
	Patient	Control
Lymphocyte subset, numbers (%)		
$CD2^+$ T cells	1041 (61)	1467–3003 (65–85)
$CD3^+$ T cells	857 (52)	1277–2929 (59–76)
$CD4^+$ T cells	171 (10)	782–1626 (32–47)
$CD8^+$ T cells	700 (50)	463–1369 (24–36)
$CD4^+/CD8^+$ ratio	(0.2)	(0.9–2.1)
$CD20^+$ B cells	290 (17)	163–411 (4.6–16.8)
$CD25^+$ T cells	(72.1)	(54–78)
DNA synthesis, cpm		
Mitogen		
PHA (10 μg/ml)	4605	67,523–122,813
Con A (10 μg/ml)	2064	21,286–62,700
Pokeweed mitogen (1:400)	2763	14,220–39,400
Antigen		
Mumps	233	4,850–24,329
Candida albicans	214	13,357–35,574
Tetanus toxoid	115	3,945–27,193
Purified protein derivative	270	0–406
Serum immunoglobulins, mg/dl		
IgG	1010	723–1685
IgM	93	63–277
IgA	88	69–382
Antibody and antigen status		
Anti-HIV-1 antibodies	Neg	Neg
HIV-1 p24 antigen	Neg	Neg
Anti-HTLV I and anti-HTLV II antibodies	Neg	Neg

Lymphocyte subsets were analyzed with monoclonal antibodies, using FACScan. $CD25^+$ (Tac/IL-2 receptor) T cells represent percent of cells positive for CD25 antigen on mononuclear cells preactivated with PHA for 3 days. Serum anti-HIV 1 antibodies were assayed by ELISA and Western blot. Serum HIV-1 p24 antigen was measured by ELISA. Serum anti-HTLV I and anti-HTLV II antibodies were assayed by an immunofluorescence technique. Neg, negative.

embedded blocks were cut at a thickness of 50–60 nm, contrast enhanced with uranyl acetate, and examined with a Philips CM 10 electron microscope.

RT Activity. Supernatants of PHA- plus rIL-2-activated MNC of the patient and H9/200 cells were collected at various time intervals and examined for precipitable RT activity. Supernatants were centrifuged at 10,000 × *g* for 15 min and treated with polyethylene glycol 8000 [30% (wt/vol) with 0.4 M NaCl]. The mixture was maintained overnight at 4°C. All specimens were centrifuged at 10,000 × *g* for 45 min at 4°C. Supernatants were discarded, and pellets were resuspended in 100 μl of buffer containing Triton X-100. Specimens were frozen at −70°C until analyzed. Negative controls consisted of supernatants of PHA- plus IL-2-activated MNC and the H9 cell line and medium alone. Positive controls consisted of supernatants from HIV-1 (Mg^{2+}-dependent RT)-infected H9 cells and simian sarcoma virus (SSV) (Mn^{2+}-dependent RT). Precipitable RT activity was measured in the presence or absence of Mn^{2+} (0.3 mM) or Mg^{2+} (10 mM) as divalent cations and dT_{12-18}·poly(rA) and dG_{12-18}·poly(rC) as synthetic primer templates (22). Data are expressed as cpm.

PCR for HIV and HTLV Proviruses. Genomic DNA was prepared by the proteinase K/phenol extraction method (23) from H9/200 cells, U1 (HIV-1-infected) cells, and MT-2 (HTLV-I-infected) cells. Genomic DNA from MNC of a normal donor was used as a negative control. One-half microgram of each DNA was used as template for PCR. Primers SK145 and SK431 were designed to amplify DNA in the *gag* region of both HIV-1 and HIV-2 proviruses. Primers SK43 and SK44 were used to amplify DNA in the *tax* region of both HTLV-I and HTLV-II proviruses. PCR was done with a thermal cycler and GeneAmp PCR kit for 42 cycles of 1 min at 94°C, 1 min at 60°C, and 3 min at 72°C. Amplified DNA was analyzed with 2% agarose gel electrophoresis. Amplified DNA was confirmed by Southern hybridization analysis with SK102 and SK45 probes for HIV and HTLV proviruses, respectively. To demonstrate the sensitivity of our PCR assay, experiments were performed with DNA isolated from U1 and MT-2 cells that were mixed with DNA from normal peripheral blood lymphocytes to obtain the final amount of DNA equivalent to that from 100%, 10%, 1%, 0.1%, and 0.01% infected cells.

Detection of Antibodies Against HICRV. Ten million H9 cells, H9/200 cells, HIV-1-infected H9 (H9/HIV-1) cells, and MT-2 (HTLV-I-infected) cells were lysed in 500 μl of lysing buffer, and nuclear materials were removed by microcentrifugation for 1 hr at 4°C. One hundred microliters of supernatant was mixed with an equal volume of sample loading buffer (2% SDS/100 mM dithiothreitol/60 mM Tris·HCl, pH 6.8/0.01% bromophenol blue), and 20 μl of the sample (≈29 μg total protein) was applied to an SDS/PAGE (10%) gel. Separated proteins were transferred to nitrocellulose paper by using a Novex (Encinitas, CA) transfer apparatus. A Western blot was probed with normal serum or the patient's serum (1:50 dilution) preadsorbed with MNC from a healthy donor. Proteins bound to serum antibodies were visualized with alkaline phosphate-conjugated goat anti-human immunoglobulin and a blot detection kit. To demonstrate that the precipitated bands in H9/200 cell lysates were not HIV-1, cell lysates were also probed with monoclonal antibodies to HIV-1 p24. The positive controls consisted of HIV-1 virus lysates and lysates from H9/HIV-1 cells. The negative control consisted of uninfected H9 cells. Antibody-bound proteins were visualized by a sequential treatment of biotinylated anti-mouse immunoglobulin and alkaline phosphatase-conjugated streptavidin, using a blot detection kit.

RESULTS

Electron Microscopy. Results of electron microscopic examination of H9 cells, H9/200 cells, and H9 cells cocultured with the daughter's MNC are shown in Fig. 1. Fig. 1 *A* and *B* shows the presence of viral particles in many cisternae of H9/200 cells. No viral particles were seen in the nucleus. The intracisternal viral particles were uniform in morphology, ≈80 nm in diameter with two concentric rings of electron-dense material and a clear center. The inner core was considerably thicker than the outer layer. Spikes were also noted on the outer surface. Twenty to 30 particles in each cell were seen in individual thin sections. Control H9 cells showed no viral particles (Fig. 1*D*). Fig. 1*C* shows the presence of similar intracisternal viral particles in cocultures of H9 cells with MNC from the patient's daughter. In addition, we have been able to transfer HICRV infection *in vitro* (as determined by electron microscopy) from the MNC of the daughter to MNC from a healthy control (data not shown). Since progressive cell death was observed in cocultures of H9/200 (without syncytia formation), it was reasoned that the virus must be released in the cultured medium. To transfer the viral infection from culture supernatants to normal MNC, supernatants from H9/200 cells were filtered and centrifuged at 10,000 × *g* for 15 min to remove cellular debris. Supernatants were then centrifuged at 40,000 × *g* for 8 hr at 4°C, and pellets were used as virus inoculum to infect PHA- plus rIL-2-activated MNC from a normal healthy donor. After 2–3 weeks in culture, cells were examined with an electron microscope. Intracisternal viral particles similar to those seen in H9/200 cells were detected (data not shown).

RT Activity. Culture supernatants from the patient's MNC stimulated with PHA plus rIL-2 were examined for precipitable RT activity in the presence or absence of divalent

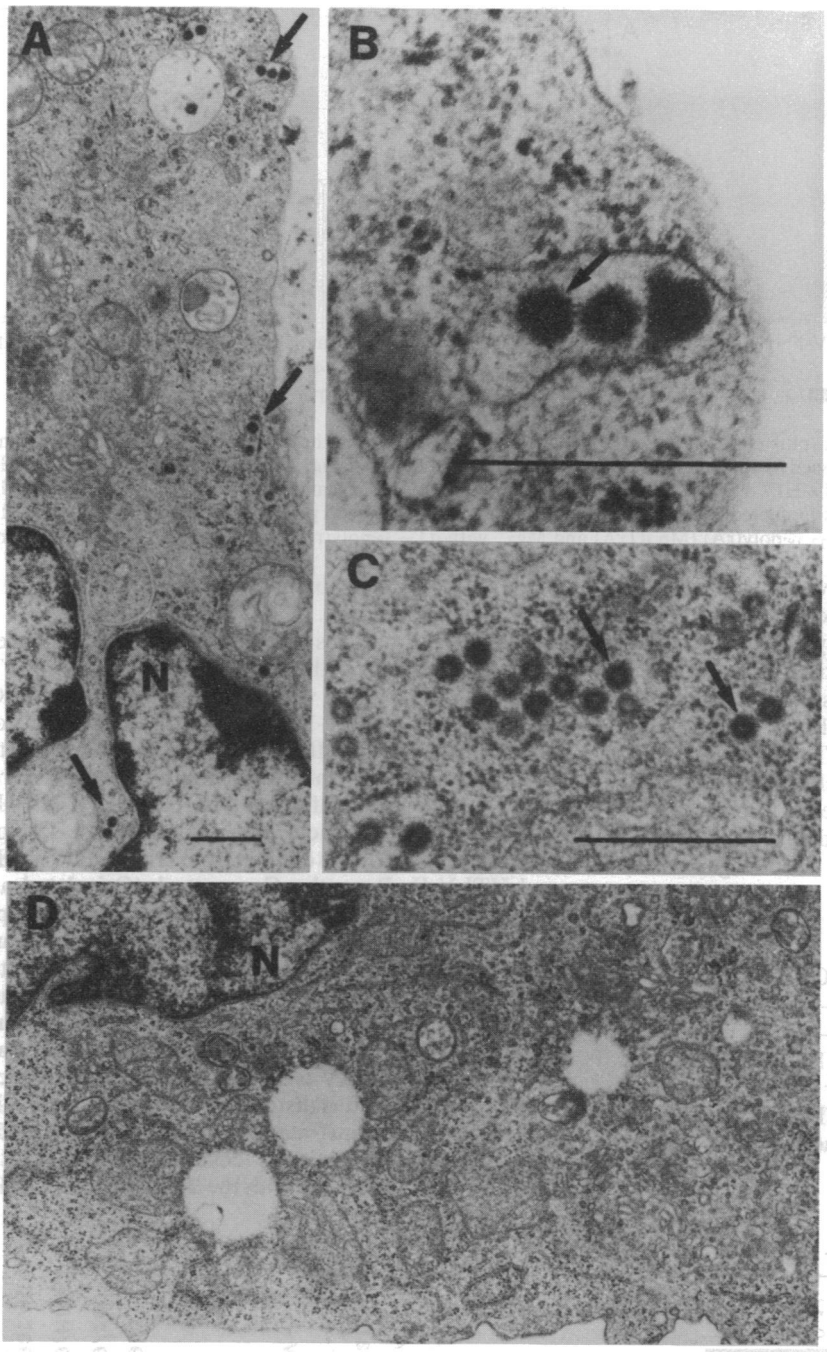

FIG. 1. Electron micrographs of H9 cells that were infected with the patient's (*A* and *B*) or the daughter's (*C*) mononuclear cells and a control noninfected H9 cell (*D*). (*A*) Part of a H9 cell that was cocultured with the patient's MNC (H9/200). Numerous virus particles appear within cisterns (arrows) in the cytoplasm but not in the nucleus (N). (*B*) Enlargement of three virus particles found in the cistern that is indicated at the top of *A*. The virus particle (arrow) displays two concentric rings of electron-dense material and a clear center. (*C*) Portion of an H9 cell that was cocultured with MNC of the patient's daughter. Note that these virus particles (arrows) have a morphology and size similar to that for those found in H9/200 cells. They are also found within cisterns. (*D*) Control H9 cell. It displays a normal-appearing nucleus (N) and a cytoplasm that lacks viral particles. (The scale bar for *D* is the same length as that in *A*; all scale bars = 0.5 μm.)

cations Mg^{2+} or Mn^{2+}, using dT_{12-18}·poly(rA) and dG_{12-18}·poly(rC) as synthetic templates. Fig. 2*A* shows a significant RT activity that was predominantly Mn^{2+} dependent. At day 7 onward, progressive cell death was observed in cultures. Similar Mn^{2+}-dependent RT activity was observed in the supernatant of HICRV-infected H9 cells (Fig. 2*B*). The positive (SSV for Mn^{2+}-dependent RT and HIV-1 for Mg^{2+}-dependent RT) and negative controls (MNC, H9, and medium) for both experiments are shown in Fig. 2*B*.

PCR. To further distinguish HICRV from HIV or HTLV, PCR analysis was performed with genomic DNA isolated from H9/200 cells, U1 cells, and MT-2 cells. DNA from a normal donor's mononuclear cells was used as a negative control. Fig. 3 shows that PCR failed to amplify HIV and HTLV proviruses from genomic DNA of the patient's MNC, whereas 142-base-pair and 159-base-pair DNA in U1 and MT-2 were amplified from HIV and HTLV proviruses, respectively. The sensitivity of our PCR technique was tested on DNA isolated from U1 and MT2 cells that were mixed with graded proportions of DNA from normal MNC to obtain DNA equivalent to 100%, 10%, 1%, 0.1%, and 0.01% of infected cells. The PCR technique was sensitive in detecting a signal at the level of 0.01% infected cells. These data suggest that HICRV is distinct from HIV-1, HIV-2, HTLV-I, and HTLV-II and that our patient is not infected with HIV or HTLV.

Serum Antibodies Against HICRV. To determine whether the patient has circulating antibodies against HICRV, her serum was examined by Western blot assay. The Western blot analysis of the patient's serum was performed by using homogenates of H9, H9/200, H9/HIV, U937/HIV-2, and MT-2 cells. A distinct band of ≈24 kDa was observed in H9/200 cells but not in homogenates of H9, H9/HIV-1, U937/HIV-2, and MT-2 cells (Fig. 4*A*). Normal serum showed no band at 24 kDa (Fig. 4*B*). To determine and exclude any possibility that the 24-kDa protein precipitated from H9/200 cells is similar to HIV-1 p24, Western blots were

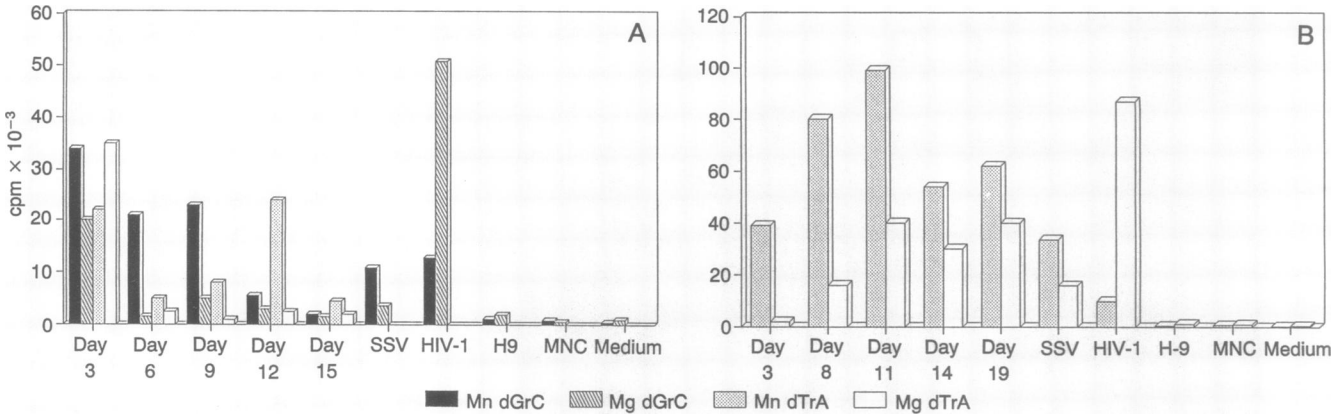

FIG. 2. RT activity in supernatants of PHA- plus rIL-2-activated MNC of the patient (*A*) and in the supernatants from cell-free transmission of HICRV-infected H9 cells (*B*). Data are expressed as cpm after subtracting the background counts. Fig. 2*A* includes negative controls (H9, MNC, and medium) and positive controls (SSV and H9/HIV-1) for Mn^{2+} with dG_{12-18}·poly(rC) (Mn dGrC) and Mg^{2+} with dG_{12-18}·poly(rC) (Mg dGrC). Fig. 2*B* shows the negative (supernatants from uninfected H9 cells, activated MNC, and medium) and positive (supernatants from HIV-1-infected H9 cells, and SSV) controls for Mn^{2+} with dT_{12-18}·poly(rA) (Mn dTrA) and Mg^{2+} with dT_{12-18}·poly(rA) (Mg dTrA) for both experiments.

performed with lysates of H9 cells (negative control), H9/200 cells, H9/HIV-1 cells (HIV-1 positive control), and HIV-1 virus lysates, using a monoclonal antibody to HIV-1 p24. No reactions were observed between the anti-HIV-1 p24 monoclonal antibody and H9/200 or H9 cell lysates, but a positive reaction was observed between anti-HIV-1 p24 and H9/HIV-1 and HIV-1 virus lysates (Fig. 5). These data demonstrate that the 24-kDa band in H9/200 cells is not shared by HIV-1 p24. Therefore, HICRV p24 appears to be antigenically distinct from that of HIV-1. The data also show the presence of antibodies in the patient's serum against the 24-kDa protein of HICRV.

DISCUSSION

In the present study we have isolated HICRV particles from a 66-year-old patient with severe $CD4^+$ T-cell deficiency and *P. carinii* pneumonia and her 38-year-old asymptomatic daughter with $CD4^+$ T-cell dysfunction. Neither the patient nor her daughter had any risk factors for HIV infection and were negative for antibodies to HIV-1, HTLV-I, and HTLV-II, and for HIV-1 p24 antigen. Furthermore, in the patient, PCR analysis failed to show infection with HIV-1, HIV-2, HTLV-I, or HTLV-II.

Human retroviruses are associated with diseases commonly associated with altered growth of $CD4^+$ T cells. HTLV-I is associated with malignant expansion of $CD4^+$ T cells (2), whereas HIV-1 is associated with depletion of $CD4^+$ T cells, resulting in AIDS (3–5). The present patient had no risk factors for HIV infection, and there was no serological or PCR evidence of infection with HIV-1, HIV-2, HTLV-I, or HTLV-II. Ultrastructurally, enzymatically, serologically, and by PCR, HICRV is distinct from HIV-1, HIV-2, HTLV-I, and HTLV-II. Recently, Garry *et al.* (6) isolated a human intracisternal A-type retroviral particle from a lymphoblastoid cell line exposed to homogenates of salivary gland from Sjögren syndrome. It is unclear whether lymphocytes from patients with Sjögren syndrome were infected with this virus. Moreover, no association with $CD4^+$ T-cell deficiency and dysfunction was reported. Although ultrastructurally and enzymatically HICRV is similar to the A-type intracisternal retroviral particle, it is distinct from the intracisternal A-type retrovirus as demonstrated by a lack of HIV-1 and HIV-2 proviruses in the patient's cells, a lack of reactivity of anti-HIV-1 p24 antibody with the 24-kDa protein of HICRV,

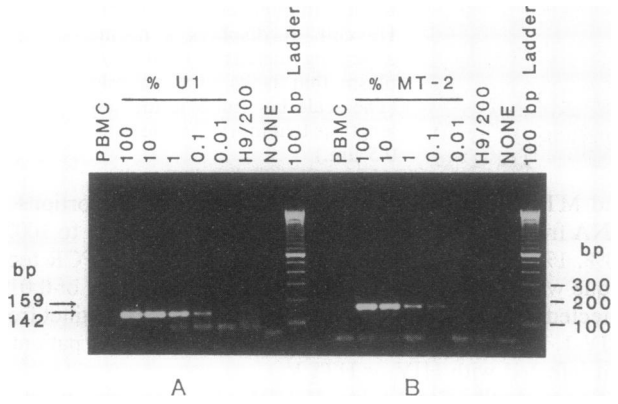

FIG. 3. PCR for HIV and HTLV proviruses. (*A*) Primers SK145 and SK431 were designed to amplify DNA in the *gag* region of both HIV-1 and HIV-2 proviruses. (*B*) Primers SK43 and SK44 were used to amplify DNA in the *tax* region of both HTLV-I and HTLV-II proviruses. No bands were observed with DNA from H9/200 and peripheral blood mononuclear cells (PBMC) from normal control and either sets of primers. Positive bands were observed with U1 and MT-2 DNA, using respective primers for HIV-1 and HTLV-I. PCR analyses were done using DNA extracted from 100%, 10%, 1%, 0.1%, and 0.01% HIV-1-infected U1 and HTLV-I-infected MT-2 to demonstrate the sensitivity of the assay. bp, base pair(s).

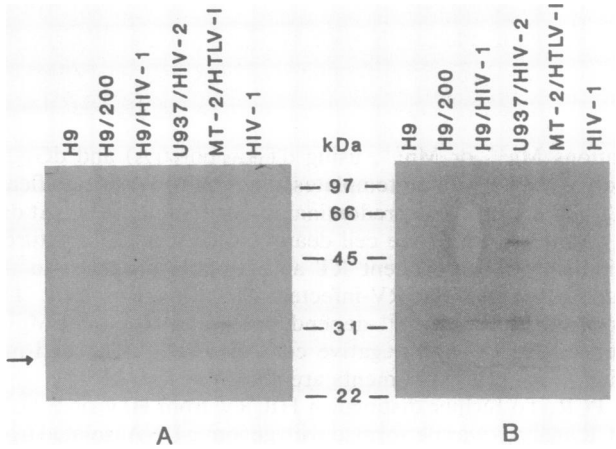

FIG. 4. Western blot analysis of patient no. 200 (*A*) and normal (*B*) serum against homogenates of H9, H9/HIV-1, MT-2, U937/HIV-2, and H9/200 cells. A distinct band of ≈24 kDa was observed between H9/200 cells and the patient's serum. The patient's serum had no such reactivity with H9, H9/HIV-1, U937/HIV-2, or MT-2 homogenates. The normal serum had no reactivity at 24 kDa with any of the cell line homogenates.

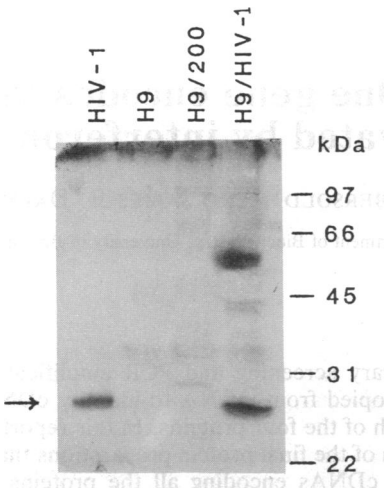

FIG. 5. Western blot analysis of anti-HIV-1 p24 monoclonal antibody against lysates from H9, H9/200, and H9/HIV-1 cells and HIV-1 virus lysates. Anti-HIV-1 p24 reacted with H9/HIV-1 cell and HIV-1 virus lysates but did not react with lysates from H9 or H9/200 cells.

and absence of serum antibodies against HIV-1 in the patient's serum. The A-type intracisternal retrovirus is antigenically related to HIV-1, and sera from patients with Sjögren syndrome are positive for HIV-1 antibodies.

In this study, we have shown the presence of circulating antibodies in the patient's serum against a protein of ≈24 kDa (likely Gag protein) by Western blot. Furthermore, this 24-kDa band is not recognized by monoclonal antibodies to HIV-1 p24, suggesting that the HICRV 24-kDa protein is antigenically distinct from that of HIV-1 p24. A cluster of patients with *P. carinii* pneumonia have been reported in HIV-1-negative subjects without any predisposing factors (18). More recently, Gautier *et al.* (19) have reported three unusual cases of *P. carinii* pneumonia associated with $CD4^+$ T-cell deficiency in whom there was no evidence of HIV-1 infection over a 1-year follow-up. Our present patient is similar to those described by Gautier *et al.* Her immunodeficiency has persisted for the past 18 months. It is possible that some of these patients with $CD4^+$ T-cell deficiency and dysfunction are infected with HICRV.

The mode of transmission of HICRV is presently unclear. A possibility of transmission of HICRV through blood transfusion is entertained. Our patient had a history of blood transfusion in 1949–1950 for bleeding following a spontaneous abortion. HICRV also appears to be vertically transmitted as demonstrated by the presence of HICRV particles in H9 cells cocultured with the MNC from the daughter of the patient. The daughter had normal proportions and numbers of $CD4^+$ T cells; however, a functional defect of $CD4^+$ T cells, as demonstrated by a poor proliferative response to soluble antigens (*Candida albicans*, mumps, and tetanus toxoid) was observed. The proliferative responses to mitogens (PHA, Con A, and pokeweed mitogen) were normal. These immunological abnormalities were reproducible over a 6-month period (data not shown). A similar immunological profile has been observed in early stages of HIV-1 infection (24, 25).

In summary, we have identified a human retrovirus (HICRV) from a patient with $CD4^+$ T-cell deficiency and *P. carinii* pneumonia and her asymptomatic daughter with $CD4^+$ T-cell dysfunction. HICRV is distinct from HIV-1, HIV-2, HTLV-I, HTLV-II, and intracisternal A-type retrovirus. The causal relationship of HICRV to $CD4^+$ T-cell deficiency remains to be established. Additional modes of transmission, cell tropism, receptor(s) for HICRV, etc. remain to be defined. A possibility of HICRV infection should be entertained in patients with $CD4^+$ T-cell deficiency and/or dysfunction, with or without opportunistic infections, in whom HIV and HTLV infections have been excluded.

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